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Enzymatic modification of triglyceride fats

Field of the invention
The present invention relates to an interesterification process
5 of triglyceride fats. More particularly the process concerns an
enzymatic interesterification process which is further denoted
as an enzymatic rearrangement process.

Background
10 Chemical interesterification of a triglyceride fat aims at an
exchange of the fatty acid residues of the glyceride moiety of
the fat. After interesterification, on the resulting
triglycerides the fatty acid residues have been exchanged by
other residues. The fatty acid residues may originate from the
15 same or from a different triglyceride molecules or they may
come from free fatty acids which were present in the reaction
mixture.

The exchange of fatty acid residues eventually results in a
20 statistically random distribution of the fatty acid residues
over the terminal and middle positions of the glyceride
molecule. The obtained fat is said to have become fully
randomised.

25 The chemical interesterification process needs a catalyst,
which usually is an alkali metal hydroxide or an alkali metal
alkanolate, such as sodium methanolate.

However, consumers increasingly prefer food and food
30 ingredients which have not been exposed to chemicals during
their preparation. Therefore a general need has arisen for non-
chemical modification processes of triglyceride fats.
Interesterification may also occur via enzymatic rearrangement.

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Such enzymatic process does not affect the naturalness of the fat.

Contrary to chemical interesterification which proceeds
5   instantaneously, enzymatic rearrangement proceeds gradually, and therefore takes more time.

For enzymatic rearrangement (ER) a lipase enzyme is used as catalyst. Lipases used for ER comprise the microbial Mucor
10 miehei lipase, Thermomyces lanuginosa lipase and Rhizopus oryzae (formerly Rhizopus delemar).

Generally, the lipases used in an ER process are sn-1 and sn-3 specific meaning that only the terminal fatty acid residues are
15 effected.

In the course of the enzymatic reaction, some randomisation at the middle position may occur. However when this happens it is due to acylmigration (see Torres et al., JAOCS vol 79, no.8
20 (2002) p775-781, Torres et al JOACS, vol 79 no 7 (2002) p655-661, and Zhang et al JAOCS vol 78, no. 1 (2001) p 57-64) which is a chemical side reaction which take place at long reaction times. The acylmigration is due to the presence of diacylglycerides which arise abundantly at long reactions times
25 and in the presence of water.

This difference in randomisation results in triglyceride products with a triglyceride composition and with properties that are quite different from the fully randomised triglyceride
30 fat resulting from chemical interesterification. Unfortunately, the extensive knowledge and experience acquired by using fully randomised chemically interesterified fats for manufacturing

food products can not be used for enzymatically interesterified
fats (Zhang et al JAOCS, Vol.78, no 1 (2001) pp. 57-64).

Furthermore the middle position in a natural feedstock fat is
5 usually is an unsaturated fatty acid, often oleic acid.
Triglycerides of the type palmitic-oleic-palmitic may cause
graininess in the fat blend. Because the middle position of the
triglycerides in an enzymatic rearrangement reaction is hardly
affected, triglycerides with a saturated middle position are
10 barely present in enzymatically rearranged fats, unless already
present in the starting material. This typical distribution of
natural fatty acids over the triacyglycerides has some
consequences. In the first place it is nutritionally beneficial
to have an unsaturated fatty acid at the middle position, since
15 the lipase activity in our digestive system delivers a 2-
monoacylgyceride and 2 free fatty acids which are derived from
the terminal triacylglycerides positions. This digestive effect
is confirmed by Nielsen (Oils and fats international (vol 18,
no 4 (2002)). He established that immobilised Lipozyme TL IM
20 action is restricted to the 1 and 3 position on the
triglyceride, leaving the middle position unaltered. However,
this configuration of fatty acids over the glycerol backbone of
the triacylglycerides also has a downside. With respect to food
structuring functionality these triacylglycerides, with a
25 unsaturated middle position, are less functional. This is due
to their lower melting point compared to fully saturated
triacylglycerides and their complicated crystallisation
behaviour.

30 As explained before, rearrangement on the middle position may
occur during enzymatic rearrangement, however in order to occur
at a appreciable amount the enzymatic rearrangement has to
proceed at equilibrium (100% conversion of sn1 and sn-3

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position) or beyond. Chemical rearrangement proceeds instantly,
meaning that instantly a complete randomisation is obtained. It
is the nature of the enzymatic reaction that in the beginning
of the reaction the conversion of sn-1 and sn3 position runs
5   quickly, but that towards equilibrium the conversion rate
proceeds more and more slowly (see figure 1). Consequently, to
attain 100% conversion long enzyme contact times are needed.

The enzymatic re-arrangement process, even though strictly sn-1
10 and sn-3 specific, is always accompanied by a some change of
the fatty acid distribution on the sn-2 position. This is due
to the unavoidable chemical process of acyl-migration that
occurs in partial fatty acid glycerides. Xu et al (Enzymatic
Production of Structured lipids: Process reactions and Acyl
15 migration, inform 11 (2000) p1121-1131) reported that
acylmigration can be attributed primarily to longer residence
times. However, the related low flow through the packed bed
reactor makes the process expensive for use on industrial
scale. (Xu et al JAOCS, Vol.79, no 6 (2002) pp. 561-565).
20 Indeed, Torres et al. recommend short reaction times to reduce
randomisation of the fatty acids residues (JOACS, vol 79, no 8
(2002) p 775-781).

Without wishing to be bound by theory the process of
25 acylmigration is independent on the enzyme used, however it is
due to the relatively slow rate of the process. Significant
effect on the middle position only occur at very high
conversion rates, often 100%, which relates to very long
contact times. This is illustrated by Fig. 3.
30

The processes reported in the prior art typically refer to time
and enzyme concentration combinations that relate to 100%
conversion of the sn-1 and sn-3 position(equilibrium) and often

in excess of the time needed to obtain 100% conversion on the
terminal positions. As a logical consequence these reactions
yield also a certain amount of randomisation of the middle
position. However, these processes are economically not

5   attractive, because of the long contact times needed to obtain
a reasonable amount of sn-2 randomisation.

For example Berben et al in society of chemical industry
(online 16 february 2001) describe a process of enzymatic

10 rearrangement wherein they have the reaction proceed until
equilibrium and obtain a randomisation on the middle position
of 18%.

WO96/14756 describes ER of fat blends using sn-1 and sn-3-

15 specific SP392 as lipase catalyst. The process is characterised
in that the rearrangement does not proceed beyond a conversion
degree of the the sn-1 and sn-3 position of 90% (but being at
least 20%), which results in shorter reaction times. However,
no randomisation at the sn-2 position is observed.

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Some rare lipases including *Candida cylindracae* and
Arthrobacter lipases are non-specific. An ER process using
those lipases delivers a fat rearranged at all glyceride
positions. However, those lipases either have been found to be

25 not suited for use at an industrial scale and/or have not been
approved for food manufacture.

The process described in EP 652289 uses a common sn-1 and sn-3-
specific lipase. The rearrangement requires the presence of a

30 substantial amount of at least 4 wt.% of diacylglycerides (also
denoted as diglycerides) in the reaction mixture. The fat
becomes rearranged at all three positions, but at the end it

contains much diglycerides and other byproducts, all of which
need to be removed by a subsequent purification process.
A cost effective ER process is needed which results in
substantial rearrangement also at the middle position. Such a
5 process would make a new range of naturally modified
triglyceride fats economically available.

It is therefore an object of the present invention to provide
an enzymatic rearrangement process wherein an appreciable
10 amount of rearrangement on the middle position occurs. A
further object of the invention is a process with a short
reaction time. Another object of the invention is to provide an
enzymatic rearrangement process without the deliberate use of
diacylglycerides.
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Surprisingly one or more of the above mentioned objects is
obtained by using a catalyst with an activity exceeding 250 IUN
(22 g/(g*h)) as measured at the onset of the process.

20 **Description of figures:**
    Fig. 1: plot of conversion degree Re vs time.
    Fig. 2: plot of randomisation on 2-position (Ra) versus contact
    time.
    Fig. 3: Plot of randomisation on 2-position (Ra) versus time
25 Re>1 indicates that the reaction time has exceeded the time
    needed to reach equilibrium with respect to the randomisation
    of the sn-1 and sn-3 positions.

    Detailed description of the invention
30 The present invention relates to a rearrangement process
    wherein the fatty acid residues on a glyceride moiety are
    randomised over the terminal and middle positions,

wherein the process proceeds to a conversion degree on the terminal positions, Re, ranging from 0.3-0.95, and wherein a conversion degree on the middle position, Ra, ranges from 0.06-0.75, and wherein Ra is greater than 0.32Re - 0.08,

the process comprises the exposure of a triglyceride fat to a catalyst comprising a lipase characterised in that the lipase is a Thermomyces lanuginosa lipase which has an activity of at least 250 IUN corresponding to 22 g/(g*h) at the onset of the process.

The IUN is a measure of the activity of the enzyme and is determined according to the procedure as described below in the experimental section.

The activity of the enzyme can also be measured with an other, more convenient method. The method measure the amount of oil converted per amount of catalyst in one hour (g/(g*h)). An activity of 250 IUN corresponds to 22 g/(g*h). The method is described in the experimental section.

The invented process has the benefit over prior art enzymatic rearrangement processes that it provides an appreciable amount of rearrangement at the middle position of the triglycerides, it does not need an excess of diacylglycerides and therefore does not need a cumbersome purification step at the end. In addition, the process of the invention has short contact times.

Conversion degree Re is the actual conversion over the sn-1 and sn-3 position. It is the conversion degree at a certain time divided by the equilibrium state (100% conversion of sn-1 and sn-3 position). Conversion degree Ra is the actual conversion on the sn-2-position only. It is the conversion of the sn-2-

position at a certain time divided by the equilibrium state at
the sn-2-position which is identical to the sn-2 fatty acid
distribution of chemically interesterified mixture.
Determination of the conversion degree Re and Ra of a

5 rearranged fat sample is based either on the change of its
carbon number profile or on the change in molar fractions of
specific types of triglycerides. The measurements are explained
in the experimental section.

10 The process of the present invention is such that even before
attaining equilibrium with respect to sn-1 and sn-3
randomisation (Re=1), a substantial randomisation of the middle
position (Ra) is achieved, ranging from 0.06-0.75. The process
of the present invention does therefore not proceed further

15 than a conversion degree Re of 0.95. This allows short
residence time such as described in WO96/14756.
The processes of the prior art proceed until equilibrium or
beyond see e.g. Berben et al in society of chemical industry
(online 16 february 2001), Torres et al., JAOCS vol 79, no.8

20 (2002) p775-781, Torres et al JOACS, vol 79 no 7 (2002) p655-
661, and Zhang et al JAOCS vol 78, no. 1 (2001) p 57-64.

With the process of the present invention long contact time and
long reaction times which belong to the processes of the prior

25 art, are avoided.
In addition, even at short contact times, i.e. low Re
conversion degree, already a minimum randomisation at the
sn-2-position occurs. For Re between 0.3 and 0.95, the
rearrangement on the middle position (Ra) is greater than

30 0.32Re - 0.08. Preferably Ra is at least 0.32Re - 0.06 and even
more preferably Ra is at least 0.32Re - 0.04.

Preferably the conversion degree Re is less than 0.9, more
preferably less than 0.85.
The conversion degree Re is preferably at least 0.35, and even
more preferably at least 0.4.

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The supplier of Lipozyme® TL IM recommends a flow of 1500 kg
oil per hour per 400 kg catalyst in a packed bed reactor. This
results in residence time of 32 minutes. A general description
of this process can also be found in Nielsen, Oils and Fat
10 international, vol 18, no 4 (2002).

In contrast the present invention where the enzyme has an
activity of at least 250 IUN corresponding to 22 g/(g*h),
allows processing of 4400 kg oil per hour employing the same
15 amount of enzyme resulting in a residence time of only 11
minutes. However, still a substantial amount of randomisation
at the middle position occurs.

Suitably during the first hour of conducting oil through a
20 packed bed reactor the residence time of the oil in the
Lipozyme® TL IM catalyst bed of the present invention
preferably is less than 25 min, more preferably less than 20
min and still more preferably less than 15 min. These residence
times are the residence times in the beginning of the
25 rearrangement when fresh catalyst is present. In the course of
the process the catalyst gradually looses activity and longer
residence times are needed for the maintenance of the degree of
conversion Re. Longer residence times may be accomplished by
reducing the flow of the oil through the catalyst bed reactor.
30 For the present invention, even when the activity of the
catalyst in the course of the reaction is reduced, and the flow
is adjusted, still the randomisation of the sn-2-position is at
an appreciable rate (see fig 2).

The present invention may also be used in a batch process.
However instead of short residence time or high oil flow, low
concentrations of a catalyst may be used. In comparison with
prior art processes where the concentration of the catalyst is
5 10 wt.%, the catalyst concentration of the present invention in
a batch process may range from 0.05 - 9 wt.%, more preferably
from 0.05 - 5 wt.% and still more preferably 0.05 - 3 wt.%
calculated on the reaction mixture.

10 The present process preferably uses the Thermomyces lanuginosa
lipase containing ER catalyst Lipozyme® TL IM which is
commercially available from NOVOZYMES, Denmark, as an aggregate
of enzyme and silica. The IUN catalyst activity is defined by
its method for measuring by NOVOZYMES, as described below in
15 the experimental section. Alternatively the activity can be
measured by another more convenient activity measurement which
is also described in the experimental section.

In the art of enzymatic rearrangement the presence of silica is
20 reported to catalyse randomisation of the middle position.
Without wishing to be bound to theory, it is thought that
silica retains water and that water hydrolyses the
triglycerides to diacylglycerides which catalyse rearrangement
at the sn-2-position. So, in the processes of the prior art,
25 more silica leads to more diacylglycerides, which leads to more
sn-2-randomisation. However, when using the highly active
lipase according to the present invention, it was surprisingly
found that less silica leads to a increase of randomisation at
the middle position.
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An economy related benefit of the present process is that it
does not need the cumbersome final purification from
diglycerides as is necessary for the process of EP 652289.

5 From the prior art the Lipozyme® TL IM catalyst is dispensed
with water and then added to the reaction mixture. The present
process however, is preferably carried out with relatively low
content of water. Preferably the amount of water is in the
range 0.001 – 0.1 wt.%, more preferably in the range of 0.001 –
10 0.05 wt.%.

Water content measurement is determined by means of standard
Karl Fischer titration, but not earlier than 30 minutes after
contacting the catalyst with the feedstock in a batch reactor.
15 When processing in a packed bed reactor, water measurement is
done in an oil sample taken downstream of the reactor but not
earlier than 30 minutes after the oil has started to flow
through the catalyst bed in order to allow for reliable
measurements.
20

Suitably the temperature of the reaction mixture is from 40 to
85°C, preferably from 45 to 80°C, more preferably from 50 to
75°C.

25 The process of the invention may be applied to a variety of
triglyceride fat blends, but is most appropriate for
triglyceride fat blends in which there is a difference in the
distribution of fatty acids over the sn-1, sn-3 and sn-2
position of the glyceride molecule.
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The process of the present invention is particularly suitable
for a feedstock comprising

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- any mixture comprising of a liquid oil and a hydrogenated
oil, preferably the hydrogenated oil is fully hydrogenated as
that leads to no trans fatty acids, or

- any triglyceride fat which has not been subjected to
5 hydrogenation, or,

- a mixture of palm fat or a palm fat fraction and a lauric fat
or a lauric fat fraction.

The mixture of palm fat or a palm fat fraction and a lauric fat
or a lauric fat fraction is preferred.

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The invention also comprises the use of an aggregate of
Thermomyces lanuginosa lipase and silica as catalyst for
partially rearranging fatty acid residues of a triglyceride fat
to a conversion degree of the terminal positions Re of 0.3 to
15 0.95, comprising a rearrangement on the middle position to a
conversion degree Ra of 0.06 to 0.75, wherein the
lipase/silica aggregate has an activity of at least 250 IUN (22
g/(g*h)), preferably at least 300 IUN (25.5 g/(g*h)), more
preferably at least 350 IUN (29 g/(g*h)).

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The invention also comprises a triglyceride fat obtainable by
enzymatic rearrangement of which the conversion degree of the
terminal positions Re is from 0.3 to 0.95 and of which the
conversion degree of the middle position of the triglyceride Ra
25 is from 0.06 to 0.75, while Ra is greater than 0.32Re - 0.08,
preferably greater than 0.32Re - 0.06, more preferably greater
than 0.32Re - 0.04.

Because the enzymatic rearrangement process of the present
30 invention is different from the often used chemical
interesterification and the enzymatic conversion process
proceeding to 100% conversion at the sn-1 and sn-3 position,
the fats resulting from the enzymatic rearrangement process of

the present invention have different properties than those
obtained by the conventional chemical interesterification and
the enzymatic conversion process proceeding to 100% conversion
on the sn-1 and sn-3 position (like Berben et al in society of
5   chemical industry (online 16 february 2001), Nielsen, Oils and
Fat international, vol 18, no 4 (2002), Torres et al., JAOCS
vol 79, no.8 (2002) p775-781, Torres et al JOACS, vol 79 no 7
(2002) p655-661, and Zhang et al JAOCS vol 78, no. 1 (2001) p
57-64).
10

Preferably the triglyceride fat is obtained by enzymatic
rearrangement of which the conversion degree of the terminal
positions Re is less than 0.9, preferably less than 0.85.
Preferably the conversion degree Re is at least 0.35,
15   preferably at least 0.4.

Fats according to the invention are suitable for the
preparation of food compositions, particularly for the
preparation of a constituting fat phase which comprises a
20   liquid oil and a structuring fat. Such fat phases are widely
used for the preparation of fat continuous emulsions used in
the manufacture of e.g. spreads.

Because an enzymatic rearrangement process qualifies as
25   natural, those fats too may be qualified as natural.

The process according to the invention allows the production of
fats enriched with triglycerides which have a saturated fatty
acid residue on the middle position. Such fats are used in food
30   applications where the crystallisation behaviour of the lipid
phase is critical for end product quality. Said
triacylglycerides strongly influence both stability of the end
products against adverse temperature conditions and

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crystallisation related process parameters based on the
intrinsic crystallisation properties of the fat.

So the process according to the invention allows the production
5 of fats like the ones in EP 831711 with low graininess despite
having a high palmitic acid content. Although the feedstock is
a blend with palm oil or a palm oil fraction, the rearranged
fat phase does not cause graininess in fat continuous emulsion
spreads prepared with such fat phase, or at least the
10 graininess is substantially reduced.

The invention also comprises the food products in which a fat
is incorporated which is obtained by the process of the present
invention.
15

The present invention has the benefit over the prior art
enzymatic rearrangement processes that it has much shorter
reaction time, thus allowing an economically feasible process.
Furthermore a substantial amount of randomisation on the sn-2-
20 position is possible with the process of the invention, while
still keeping the naturalness of the fat. In addition, because
there is already a substantial amount of randomisation of the
middle position at short reaction time and low conversion
degree on the sn-1 and sn-3 position, the rearrangement process
25 can be stopped at various times, resulting in fats with
different properties. One may choose the conversion degree, Re
and Ra, by stopping the reaction at a certain time and thereby
fine-tuning the property of the obtained fat.

15

Experimental section

Determination of residence time.
Residence time in hours is determined by taking the volume of
5 the catalyst bed inclusive oil, subtracting the volume of the
catalyst and dividing the difference by the volume of oil
passing the bed in one hour.

10 Establishing the IUN activity of lipase catalyst

1. PRINCIPLE
The method is provided by the catalyst supplier and is based on
15 interesterification of triglycerides by an immobilised lipase.
The conversion of tristearin in a substrate composed of 27 w/w
% fully hydrogenated soy bean oil and 73 w/w % refined,
bleached and deodorized soy bean oil at 70 °C and 200 rpm
stirring is used to quantify the activity of the catalyst. The
20 concentration of tristearin is determined using an HPLC based
analytical method.

2. SPECIFICITY AND SENSITIVITY
Components having the same retention time as tristearin in the
25 chromatographic method used will cause an overestimation of the
tristearin concentration.

3. DEFINITION OF UNITS
The interesterification activity is defined as the initial
30 conversion rate for tristearin at standard conditions. 1 IUN
corresponds to a conversion rate of 0.01 g tristearin/l
/minute/ gram catalyst.

4. APPARATUS

Analytical balance

Shaker water bath

Pipettes   Positive displacement pipette for taking oil

5   samples (due to high viscosity of the liquid).

Standard pipettes (e.g. Finnpipette) for the

other steps in the analysis.

HPLC system including the following modules: HPLC pump,

10 injection system and column heater.

Light scattering detector, e.g. Sedex 55.

HPLC Column RP 18e (5µm), LiChroCart 250-4.

Data acquisition   Data can be acquired and processed using

chromatography software.

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ANALYTICAL CONDITIONS

Column temp.:   40°C

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Flow: 1.5 mL/min.

Mobile phase: The mobile phase consists of 50 v/v %
                       acetonitrile, HPLC grade and 50 v/v %
25   dichloromethane, HPLC grade.

Injection volume: 20 µL.

Run time: At least 11 minutes.

30

Column temperature: 40°C (±2°C)

Detector: 40°C (±2°C), nitrogen pressure 2.3 bar.

35 Detector sensitivity: Gain 6

5. REAGENTS AND SUBSTRATES

CHEMICALS

Tristearin. SIGMA grade, Approx. 99%

 5 SOLUTIONS

Substrate (20 grams):

27 w/w % fully hydrogenated soy bean oil (delivered by ADM, Illinois, USA)

73 w/w % refined, bleached and deodorised soy bean oil

10 (delivered by ADM, Illinois, USA)

Catalyst (2 gram): The samples are conditioned at a_w =0.3 for at least 24 hours.

15 **6. SAMPLES AND STANDARDS**

STANDARDS

A standard curve of tristearin is made in the concentration range from 0.25 to 2.0 mg/ml.

20 LEVEL CONTROL

Reference sample: Lipozyme® TL IM reference PPW6503-3, particle size fraction 425-500μm, is used (single determination).

25 **7. PROCEDURE**

As substrate a blend of 27 w/w % fully hydrogenated soy bean oil (FH SBO) and 73 w/w % refined, bleached and deodorised soy bean oil (RBD SBO) is made. The oil mixture is heated to 80 °C in a water bath, and is well mixed.

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The oil mixture is weighed out in 100 ml conical flask with a screw top wail, about 20 grams in each. The precise weight is

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notated with 2 decimals. The flasks (batches) are placed in the
shaker water bath at 70°C and 200 rpm.

A catalyst amount corresponding to approx. 10% of the amount
5 of oil is weighted out (approx. 2 grams). The precise weight of
the catalyst is determined with 2 decimals.

When the oil mixture is homogeneous, a time zero-sample is
taken. 100µl of the oil is taken with a positive displacement
10 pipette (Gilson microman). The pipette tip is wiped off by a
Kleenex to remove outside oil mixture and the sample is
deposited in a HPLC vial (type BROWN 12x32mm with Silicone/PTFE
Septa).

15 The weighed amount of catalyst is added to the flasks
containing the oil mixture and samples are taken to the times:
15, 30, 45 and 60 minutes. All samples are 100 µl. The samples
are stored in a freezer until HPLC analysis.

20 The samples in the HPLC vials are diluted with 900 µl
dichloromethane. This solution is mixed on a whirl-mixer,
before a further dilution of 100 µl to 900 µl dichloromethane
is made (total dilution of 100x). After a further mixing this
sample is being analysed by HPLC.
25

The diluted samples are analysed by HPLC-ELSD.

The response versus concentration of the tristearin standards
are fitted to an exponential model.
30

8. CALCULATION

The concentration of tristearin in the samples is calculated by use of the standard curve.

5   The conversion of (the decrease in) tristearin concentration versus time is fitted to a exponential model, by non linear parameter estimation. The model is:

$$C_{Tristearin}(t) = C_{0,est} \cdot \exp(-k_{est} \cdot t)$$

10 Where

$C_{tristearin}(t)$ = is the concentration of tristearin in the reaction
15   mixture at time, t.

$C_{0,est}$ = start concentration of tristearin (estimated parameter)

k_{est} = rate constant (estimated parameter)

t   = reaction time

20

From the estimated rate constant, k_{est}, the activity at standard conditions, the IUN activity, can be calculated according to the following formula:

$$IUN/g = k_{est} \cdot C_{0,std} \cdot 100 \cdot \frac{W_{std}}{W} \cdot \frac{M_{oil}}{M_{oil,std}}$$

25 $C_{0,std}$ = start concentration of tristearin at standard condition.

W_{std} = weight of catalyst under standard conditions (2g)

W   = actual weight of catalyst

M_{oil} = actual weight of oil

30 $M_{oil,std}$ = weight of oil under standard conditions (20g)

Determination of activity in g/(g*h)

Calculating activity

5   *Substrates:*

50g refined bleached and deodorised quality dry fractionated
palm stearin fraction mp 53
50g refined bleached and deodorised quality palm kernel oil
an amount of immobilised enzyme catalyst such that a conversion

10 degree Re between 0.2 and 0.4 is obtained at approximately one
hour. Some experiments with different amounts of enzyme is
usually sufficient to find the right amount of enzyme. For an
enzyme with an activity of 455 IUN/38 g/(g*h) a conversion
degree Re between 0.2 and 0.4 is obtained at approximately 1

15 hour with an amount of 1 wt%.

Procedure:

The two oils are mixed together well in a closed 100ml reaction
vessel and brought to 70°C under agitation. The enzyme catalyst

20 is added to the oil substrate and the mixture agitated at 70°C.
A sample of oil is taken after 1 hour, ensuring that no enzyme
catalyst is present, and then every hour until the reaction is
at equilibrium.

25 A plot is made of conversion degree Re against time as shown in
figure 1.

The activity is then determined according to the following
equation:

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$$\text{Activity} = -\ln(1-x) * \frac{\text{amount of oil (g)}}{(\text{amount cat (g)} * t \text{ (h)})}$$

where,

x is the conversion degree, Re (determined as described) and
is a point after approximately 1 hour when Re is between 0.2
and Re=0.4. However Re should never be above 0.4.

5   t is the time
the amount of oil and catalyst is in grams, and are known
parameters.

Care should be taken that the point x is taken on the linear
part of the curve and not too close to the starting point.

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Determination of Re and Ra

Determination of the conversion degree Re of a rearranged fat
sample is based either on the change of its carbon number

15 profile or on the change in molar fractions of specific types
of triglycerides. The determination proceeds as follows:

For a to be rearranged fat sample the overall fatty acids
composition, the composition of fatty acids on the middle

20 position and the triglyceride composition is analysed. Common
analysis methods are used comprising FAME-analysis, the
GLC/carbon number method and the HPLC/silver phase method as
are described in for example EP 78568, EP 652289, JAOCS,
(1991), 68(5), 289-293 and Hammond E.W.J., Chromatography, 203,

25 397, 1981.

The molar fraction ($pA_{sn1,3}$) of any specific fatty acid residue
(A) on a terminal position (either the sn-1 or the sn-3
position, which positions are assumed to be identical) is

30 calculated according to the following formula:

$$pA_{sn1,3} = (3 * pA_{total} - 1 * pA_{sn2}) / 2$$

where pA_{total} denotes the total occurrence of a particular fatty acid residue A in the fat blend and pA_{sn2} the occurrence at the 2-position of fatty acid residue A situated. For all occurring fatty acid residues having a molar fraction larger than 0.002

5 the value $pA_{sn1,3}$ is established. The distribution of these molar fractions should be normalized to 1.0.

The triacylglyceride profile of a fully randomised triglyceride fat is calculated by simple statistics known to the man skilled

10 in the art. The mole fraction p(ABB) of the triacylglyceride ABB, for example, is calculated using the formula: $p(ABB) = 2 * pA_{sn1,3} * pB_{sn2} * pB_{sn1,3}$

Generally, the carbon number of a specific triglyceride

15 molecule is the total number of carbon atoms on its three fatty acid residues. The carbon number profile of a particular fat blend consists of the occurrence percentages of all carbon numbers of that fat blend.

20 The carbon number profile of a fat blend is derived from its triacylglyceride composition (the mole fraction collection of all triacylglycerides).

From the triacylglycerol profile of the fat blend the carbon

25 number profile can be derived easily.

By analysis the carbon number profile is established for the starting fat blend and for a specific partially rearranged sample taken from a reaction mixture. The carbon number profile

30 of the theoretically sn-1 and sn-3 fully randomised fat blend is found by a statistical calculation of the triglycerides profile as described above.

The degree of conversion of the partially rearranged fat sample
is calculated as follows:
For each carbon number in the range 30 - 60 the differences are
5 calculated between the mole fraction at the beginning of the
reaction and the mole fraction at full sn-1 and sn-3
randomisation (equilibrium). The sum of the (absolute) values
of these differences (100% absolute change of carbon numbers)
defines 100% rearrangement of the fat blend.
10

In the same way for a specific sample taken from a reaction
mixture for each carbon number between 30 to 60 the differences
are calculated of the mole fraction at the begin of the
reaction and the actual mole fraction when sampling the
15 reaction product. Again the absolute values of these
differences are accumulated. The sum is the actual absolute
change of carbon numbers until the moment of sampling.

For the specific sample the conversion degree Re = (actual
20 absolute change of carbon numbers) / (100% absolute change of
carbon numbers).

This equation applies for triglyceride products where the
abovementioned 100% absolute change of carbon numbers is at
25 least 0.15. If not, the degree of conversion Re should be
determined in an alternative way:

For each triacylglyceride of the type H3, H2O and H2L (where H
indicates fatty acid residues of palmitic or stearic acid, O of
30 oleic acid and L of linoleic acic) the mole fraction is
established by analysis. For each of these triglycerides the
absolute change between the molar fraction at the beginning of
the reaction and at full sn-1 and sn-3 randomisation is

calculated. The sum of the absolute values of these changes belongs to the state of 100% conversion.

For a specific sample taken during the proceeding reaction the
5 difference between the mole fraction at the begin of the reaction and at the actual state of the reaction product is calculated for the same selected triglycerides. The absolute values of the differences in mole fraction are accumulated. The sum defines 100% rearrangement of the fat blend.
10
The degree of conversion Re of the specific sample follows from the equation:

Re = (actual change of triacylglycerides) / (100% absolute
15 change of triacylglycerides).

Determination of Ra, the degree of rearrangement at the middle position, proceeds as follows:

20 For the initial feedstock and for a sample taken from the reaction mixture first the total occurrence of fatty acid residues and the occurrence of fatty acid residues situated at the 2-position of the triacylglyceride is established using GLC-FAME and 2-position analysis. For methods see the
25 references described above.

For each fatty acid occurring in the triacylglyceride fat at a molar level of at least 0.002 the absolute difference of its Sn-2 molar fraction at the beginning of the reaction and its
30 molar fraction in the reaction mixture is calculated, wherein the latter mixture is equal to chemically randomised fat. The sum of the absolute values of these changes (Fa-Sn2-100%) for

all selected fatty acids defines the status of 100% degree of
Sn-2 randomness.

For a sample taken during the proceeding reaction in the same
5 way the absolute change between the Sn-2 molar fraction at the
begin of the reaction and at the actual state of the reaction
product is calculated for each of the selected fatty acids. The
sum of the absolute values of these changes (Fa-Sn2-actual) is
the actual absolute change of fatty acids on the Sn-2 position.
10

For the specific sample the Ra value follows from the equation:
  Ra = (Fa-Sn2-actual) / (Fa-Sn2-100%)

Example 1

15

60 g of palm oil and 40 g of palm kernel oil were mixed
together in a 100 ml reaction vessel and heated to 70°C. 1 wt.%
of Lipozyme® TL IM (activity 455 IUN, 38 g/(g*h)) was added to
20 the mixture and this was stirred at 70°C. Samples were taken at
intervals and the degree of conversion Re and the degree of
conversion Ra were determined according to the methods
described elsewhere in this specification by means of
establishing changes in carbon number distribution and changes
25 in composition of fatty acids on the middle position.
At the conversion degrees Re of 0.85 and 0.5, the following
degrees of rearrangement of the middle position have been
found:

TABLE I

Degree of conversion Re	Degree of conversion Ra
0.85	0.28
0.5	0.1

30

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Example 2

Example 1 has been repeated but using a low activity Lipozyme®
TL IM lipase catalyst and the 10% catalyst concentration as
5 used in the prior art.

Example 3

Example 1 has been repeated using the same high activity
Lipozyme® TL IM catalyst, but with the same high catalyst
10 concentration as used in comparison example 2, allowing a
contact time which is much shorter than necessary in examples 1
and 2.

TABLE II

ER using Lipozyme® TL IM catalyst	Example 1	Example 2	Example 3
Catalyst conc. (wt.%)	1	10	10
Water (wt.%)	0.013	0.025	0.024
Reaction time (h)	5.25	2.75	0.60
Activity (IUN)	455	160	455
Activity g/(g*h)	38	18	38
Conversion Re	0.85	0.85	0.85
Randomness Ra	0.28	0.08	0.21
Ra > 0.32Re - 0.08	yes	no	yes
Ra > 0.32Re - 0.06	yes	no	equal
Ra > 0.32Re - 0.04	yes	no	no

15 The high activity Lipozyme® TL IM catalyst (example 1) causes
at a low concentration the same conversion Re as the prior art
low activity catalyst of example 2 but accompanied by a high
rearrangement of the middle composition. When increasing the
catalyst concentration according to example 3 the same effect
20 as example 1 is obtained with a shorter contact time, which
enables a higher throughput.

27

Examples 4 - 6

Examples 1 - 3 have been repeated under the same conditions, respectively, but using shorter contact times. The shorter
5 contact times result in a lower degree of conversion Re, but even at these lower conversion degrees the same relatively high rearrangement on the middle position is observed.

TABLE III

ER using Lipozyme® TL IM catalyst	Example 4	Example 5	Example 6
Catalyst conc. (wt.%)	1	10	10
Water (wt.%)	0.013	0.025	0.024
Activity (IUN)	455	160	455
Activity g/(g*h)	38	18	38
Conversion Re	0.4	0.4	0.4
Randomness Ra	0.08	0.02	0.08
Ra > 0.32Re - 0.08	yes	no	yes
Ra > 0.32Re - 0.06	yes	no	yes
Ra > 0.32Re - 0.04	no	no	no

10

Example 7 Graininess in spreads

Hardstock A was prepared by the following method:

50wt % of palm oil and 50wt % of palm kernel oil were mixed
15 together in a reaction vessel and 0.53 wt% of Lipozyme® TL IM (activity 38 g/(g*h)) was added to the mixture. This was stirred at 70 °C until a conversion Re was reached of 0.63. Rearrangement of the sn-2 position Ra was determined as being 0.18.

20

28

A fatblend was prepared from the following:

- 42 wt% hardstock A
- 5 wt% of interesterified dry fractionated palm oil stearin
5 (62 wt%) with palm kernel oil (38wt%)
- 53 wt% rapeseed oil

A fat phase was prepared by mixing 99.7 parts of fatblend, 0.2
parts of lecithin and 0.1 parts of monoglyceride (Hymono 8903).
10 An aqueous phase was prepared from 96.3 parts water, 2.2 parts
sour whey powder and 1.5 parts salt. The pH was adjusted to 4.6
by means of addition of citric acid.
80 parts fat phase and 20 parts aqueous phase were combined and
processed in a conventional manner using a votator, to obtain a
15 spread which is packed in tubs. The spreads were produced
using an AAC sequence. The temperature after the second A-unit
was 8°C and after the C-unit was 16°C. The A-units were
operated at 600rpm and the C-unit at 230rpm. The residence time
in the C-unit was approximately 90 sec. The product was stored
20 at 5°C for 5 weeks.

For comparison, margarines were made using a fatblend in which:
i.   Hardstock B (Re 0.62, Ra 0.10) was used, which was
       prepared with Lipozyme® TM IM of activity 18 g/(g*h)
25 ii.   Hardstock C (Re 0.60, Ra 0.05) was used which was prepared
       with Lipase D (Rhizopus oryzae on Accurel carrier).

Everything else was kept the same.

30 An expert panel judged the products for the presence of
tropical grains.

In margarines which had been made with hardstock A, grains were scarce and barely noticeable.

In margarines which had been made with hardstock B, grains were apparent at unacceptable levels creating an intolerable product defect.

In margarines which had been made with hardstock C, grains were clearly abundant throughout the product and thus a clear unacceptable defect.

Table IV

	Enzyme catalyst		
	Lipozyme® TL IM (hardstock A)	Lipozyme® TL IM (hardstock B)	Lipase D (hardstock C)
Activity (g/(g*h)) IUN	38 455	18 160	53 –
Concentration wt%	0.53	1.03	0.34
Re	0.63	0.62	0.60
Ra	0.18	0.10	0.05
Presence of grains in final product	Scarce, acceptable	Numerous, unacceptable	Abundant, unacceptable

In the product made with the process of the present invention hardstock A, where Ra is highest, no or hardly any tropical grains were detected. On the contrary, products made with either a low activity (hardstock B) or a sn-1 and sn-3 specific lipase (hardstock C) give rise to unacceptable levels of tropical grains.

Example 8: Graininess

60 wt% of palm oil and 40 wt% of palm kernel oil were mixed together in a reaction vessel and 0.9 wt% of Lipozyme® TL IM (activity 455 IUN, 38 g/(g*h)) was added to the mixture. This

30

was stirred at 70 °C to give hardstock A with Re 0.68. Ra was
determined to be 0.22. Harstock B was made with Re 0.26 and Ra
0.08 and 0.6 wt% Lipozyme® TL IM (activity 455 IUN, 38
g/(g*h)).

5

A fatblend was prepared from the following:
42 wt% hardstock A
5 wt% interesterified palm stearin with palm kernel oil
53 wt% rapeseed oil

10

A fat phase was prepared by mixing 0.9971 parts of fatblend,
0.0020 parts of lecithin and 0.0009 parts of monoglyceride.
An aqueous phase was prepared from 0.9630 parts water, 0.0220
parts sour whey powder and 0.0150 parts salt and citric acid to

15   a pH of 3.8.
80 parts fat phase and 20 parts aqueous phase were combined and
processed in a conventional manner using a votator, to obtain a
spread which is packed in tubs. The spreads were produced
using an ACAA sequence. The temperature after the first A-unit

20   was 15°C, after the C-unit was 18°C and after the last A unit
was 10°C. The A-units were operated at 600rpm and the C-unit at
200rpm. The residence time in the C-unit was approximately 90
sec. The product was stored at 5°C for 5 weeks. No tropical
grains developed in the product.

25

For comparison, margarines were made using a fatblend in which
Hardstock B was used.

In products in which hardstock A was used, no tropical grains

30   had developed.
In products in which hardstocks B were used, tropical grains
were identified after 5 weeks of storage at 5°C.

Table V

	Hardstock	
	A	B
Activity IUN g/(g*h)	455 38	455 38
Re	0.68	0.26
Ra	0.22	0.08
Tropical grains	No	Yes

Example 9: Ra vs reaction time

5 Following the same procedure as outlined in example 1, four
experiments in a batch reactor were performed. In table IV the
type of enzyme, its activity, and its concentration in the
reactor are given.

Table VI

experiment	Lipase	Concentration (w/w) %	Activity g/g/h
1	Lipozyme® TL IM	2.4	38
2	Lipozyme® TL IM	10	38
3 comparative	Lipozyme® TL IM	10	18
4 comparative	Lipase D	10	53

10

The reaction mixture again consists of 60 parts palm oil and 40
parts palm kernel fat. The four reactions were monitored
closely over time. A number of samples were taken and analysed.
The last sample was taken after 10 hours reaction time. From
15 the analytical data the degree of conversion (Re) and the
randomisation of the sn-2 position (Ra) were derived as
outlined in the text. Figure 3 illustrates the evolution of Re
and Ra over time for the different reactions.

32

The graph clearly shows, that for the Lipase D (experiment 4) and the low activity Lipozyme® TL IM (experiment 3) the small changes in the composition of the sn-2 position are progressing linear with time. This is in line with the prior art stating

5   the effect of acyl migration in partial fatty acid glycerides (see Torres et al., JAOCS vol 79, no.8 (2002) p775-781, Torres et al JOACS, vol 79 no 7 (2002) p655-661, and Zhang et al JAOCS vol 78, no. 1 (2001) p 57-64).

10 In contrast to this, Lipozyme® TL IM with a high activity and a concentration level of 10 (w/w) % (experiment 2) yields a dramatically increased randomisation of the sn-2 position at the same reaction time.
    It should be pointed out that in comparison to the experiment 4

15 (lipase D) these timings are actually corresponding to practically identical degrees of conversion with respect to the randomisation of the sn-1 and sn-3 positions (Re). The different shapes of the curves also illustrate this.

20 Also experiment 1, with a lower Lipozyme® TL IM concentration shows a clearly different behaviour than the the comparative experiments (3 and 4). When comparing the experiments 1 and 2 one finds that for a given degree of conversion (the randomisation of the sn-1 and sn-3 positions (Re)) the

25 experiment employing a low concentration of the enzyme catalyst actually yields higher degrees of randomisation of the sn-2 position (Ra). This increase in Ra is coinciding with the presence of less silica in the reaction vessel.

30 As this example clearly demonstrates, the process we have found is substantially different from the processes reported in the prior art.

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Example 10: RA vs contact time

60 parts of palm oil is mixed well with 40 parts of palm kernel
oil at 70°C and added to the feed tank of a packed bed reactor
(PBR) system, of which the temperature is also 70°C.

5 A PBR of 20ml volume is filled with 3g of Lipozyme® TL IM
(Activity IUN 455, 38 g/(g*h)) to give a catalyst bed volume of
7ml.

The flow meter, controlling the oil flow through the reactor is
set to 35g/h, which corresponds to a residence time of around 8
10 minutes, in order to achieve an Re of approximately 0.6.
Samples are taken at regular intervals and the flow adjusted
accordingly in order to compensate for the deactivation of the
enzyme and thus retain an Re of 0.6.

Table VII

Relative activity (%)	Re	Ra	Flow (g/h)	Residence time (min)
100	0.58	0.15	35	8
42	0.63	0.15	13	22
32	0.62	0.14	10	28
9	0.64	0.12	3	105

15

In the same way, a second experiment was carried out whereby
the flow was set to 20 g/h, which corresponds to a residence
time of 14 minutes, in order to achieve a Re of approximately
20 0.8.

Table VIII

Relative activity (%)	Re	Ra	Flow (g/h)	Residence time (min)
100	0.84	0.30	20	14
77	0.83	0.29	16	17
47	0.83	0.27	10	28
8	0.84	0.25	2	165

The results documented in the tables VII and VIII above,
25 illustrate that in order to maintain a constant degree of

34

conversion over time in a packed bed flow reactor, it is necessary to adjust the flow rate in a reciprocal way to the deactivation of the enzyme catalyst (reduction of activity). To our surprise, we have found that the resulting randomisation of

5 the sn-2 position (Ra) is practically not affected by these dramatically increased residence times, from 14 to 165 minutes. If the found randomisation would be the result of the often-cited acyl migration in partial fatty acid glycerides, the sn-2 randomisation (Ra) should increase dramatically with increasing

10 residence time. The process according to the invention, however, yields that the sn-2 randomisation (Ra) is practically constant when the residence time is increased.

The data also show that the sn-2 randomisation (Ra) is not

15 changing when an enzyme catalyst with high starting activity, according to the invention, is as a consequence of the ongoing process functioning at a reduced activity. This proves that the high starting activity of the enzyme catalyst, as defined in the text, is the key element to the establishment of the sn-2

20 randomisation (Ra).